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# **MAKING SENSE FROM NONSENSE AND MISSENSE: PHARMACOLOGICAL RESCUE OF MUTANT TUMOR SUPPRESSOR P53**

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# Making sense from nonsense and missense: pharmacological rescue of mutant tumor suppressor p53 THESIS FOR DOCTORAL DEGREE (Ph.D.)

## **Academic dissertation**

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*To my beloved family*  
致我亲爱的家人



## ABSTRACT

A large fraction of human tumors carry inactivating mutations in the *TP53* tumor suppressor gene. Approximately 75% of these mutations are missense mutations and around 10% are nonsense mutations. *TP53* inactivation allows evasion of cell death and rapid tumor progression. Restoration of wild type p53 protein expression in tumor cells can suppress tumor growth in vivo. Missense mutant p53 is often expressed at high levels in tumors. Therefore, mutant p53 is a promising target for novel targeted cancer therapy.

APR-246 (PRIMA-1Met) is a low molecular compound previously discovered in our group. We have shown that APR-246 is converted to methylene quinuclidinone (MQ), a potent electrophile and Michael acceptor, binding covalently to cysteines in p53. MQ promotes refolding of mutant p53 to wild type conformation and restores normal function to mutant p53, resulting in induction of apoptosis and inhibition of xenograft tumor growth in mice. However, the underlying mechanism is not fully elucidated. APR-246 is currently tested in a phase II clinical trial in high-grade serous ovarian cancer.

In paper I, we show that APR-246, via MQ, targets the Sec residue in the redox enzyme TrxR1 and inhibits the reducing activity of both recombinant TrxR1 in vitro and cellular TrxR1 in cells independently of p53 status. The inhibited TrxR1 maintains its ability to oxidase NADPH, which contributes to oxidative stress and cell death induced by APR-246.

In paper II, we have confirmed the ability of aminoglycosides to induce translational readthrough of nonsense mutant *TP53* and also shown that combination treatment with aminoglycosides and p53-Mdm2 inhibitors such as nutlin can enhance levels of full-length p53 and p53 activity in tumor cells carrying *TP53* nonsense mutation.

In paper III, we through data mining have identified 28 compounds with potential ability to target nonsense mutant *TP53*-carrying cancer cells. Among these, the known anticancer drug 5-FU and 5 other compounds were shown to induce full-length p53 protein in nonsense mutant *TP53*-carrying cancer cells. Full-length p53 protein induced by 5-FU is transcriptionally active as assessed by upregulation of several p53 target genes.

In paper IV, we identified 65 compounds, from high-throughput screening and FACS-based screening of chemical libraries as candidate readthrough-inducing compounds (CRICs). Among these, 7 compounds were shown by Western blotting to induce full-length p53 protein in nonsense mutant *TP53*-carrying tumor cells.

In conclusion, our study reveals that the missense mutant p53-reactivating compound APR-246 inhibits TrxR1 and converts the enzyme to an NADPH oxidase. We have also shown that combination treatment with p53-Mdm2 inhibitors enhances the ability of aminoglycosides to restore expression and function of full-length p53 in cancer cells with nonsense mutant *TP53*. Furthermore, we identified 5-FU and several novel compounds as compounds that can rescue expression of full-length p53 in tumor cells with nonsense mutant *TP53*. Our studies shed further light on the mechanism underlying APR-246 induced cancer cell death and raise possibilities for nonsense mutant *TP53*-targeted cancer therapy in the future. Our results may ultimately facilitate the development of novel treatment for tumors carrying missense or nonsense mutant *TP53*.

## LIST OF SCIENTIFIC PAPERS

- I. Peng, X.\*, **Zhang, M.Q.\***, Conserva, F.\*, Hosny, G., Selivanova, G., Bykov, V.J., Arner, E.S., and Wiman, K.G.  
APR-246/PRIMA-1MET inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase.  
*Cell Death Dis* (2013) 4, e881. (\*these authors contributed equally).
- II. **Zhang, M.\***, Heldin, A.\*, Palomar-Siles, M., Ohlin, S., Bykov, V.J.N., and Wiman, K.G.  
Synergistic rescue of nonsense mutant tumor suppressor p53 by combination treatment with aminoglycosides and Mdm2 inhibitors.  
*Front Oncol* (2017)7, 323. (\*these authors contributed equally).
- III. **Zhang, M.**, Palomar-Siles, M., Heldin, A., Bykov, V.J.N., and Wiman, K.G.  
Rescue of nonsense mutant p53 in human tumor cells by compounds identified through data mining (Manuscript).
- IV. **Zhang, M.**, Heldin, A., Bykov, V.J.N., and Wiman, K.G.  
High-throughput screening for identification of novel compounds that induce translational readthrough of nonsense mutant TP53 (Manuscript).



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## LIST OF ABBREVIATIONS

aa	amino acids
AIF	apoptosis-inducing factor
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and rad3 related
CPT1C	carnitine palmitoyltransferase 1C
CRIC	candidate readthrough-inducing compound
DBD	DNA binding domain
FADD	FAS-associated death domain
GPX4	glutathione peroxidase 4
GLS2	glutaminase 2
G6PDH	glucose-6-phosphate dehydrogenase
GI	growth-inhibition
HDM2	human double-minute 2
HPV	human papillomavirus
iPSCs	induced pluripotent stem cells
LIF	leukemia inhibitory factor
MDM2	mouse double-minute 2
MDM4	mouse double-minute 4
MQ	methylene quinuclidinone
NMD	nonsense-mediated decay
NLS	nuclear localization signal
NCI	National Cancer Institute
OD	oligomerisation domain
PRD	proline-rich domain
PRIMA-1	P53 reactivation and induction of massive apoptosis
PTMs	post-translational modifications
PTC	premature termination codon
RD	regulation domain
RITA	reactivating p53 and inducing tumor apoptosis
ROS	reactive oxygen species

SREBP	sterol regulatory element-binding proteins
SCO2	cytochrome <i>c</i> oxidase subunit 2
TA	transactivation domain
TrxR1	thioredoxin reductase 1
UV	ultraviolet
5-FU	5-fluorouracil

# **1 INTRODUCTION**

## **1.1 CANCER**

Cancer, one of the leading causes of morbidity and mortality worldwide, is a collection of diseases involving abnormal and uncontrolled cell growth. Malignant tumors invade and spread to other organs and form distant metastasis. Cancers, classified according to their primary site of origin, can arise from any tissues and any specialized cell types throughout the body (Weinberg, 2007; Torre et al., 2015). Approximately 14.1 million new cases of cancer occurred worldwide 2012, and an estimated 23.6 million new cases in 2030 worldwide (Bray et al., 2012).

There are more than 100 types of cancers, such as brain cancer (glioma), breast cancer, colon cancer, and renal cancer. All these different types of cancers share 10 common features defined as the Hallmarks of cancer: 1) sustaining abnormal proliferative signaling; 2) evading growth suppressors such as inactivation of tumor suppressors p53 and Rb; 3) avoiding immune destruction; 4) enabling unlimited replicative potential; 5) tumor-promoting inflammation supplying bioactive molecules to the tumor microenvironment contribute to multiple hallmark capabilities; 6) activating invasion and metastasis with the consequence that cancers spread to other organs; 7) inducing angiogenesis to better supply tumors with nutrients and oxygen; 8) genome instability and mutation; 9) resisting cell death, for example programmed cell death (apoptosis); and 10) abnormal cellular energy metabolism (Hanahan and Weinberg, 2011).

## **1.2 ANTI-CANCER THERAPY**

Surgery, radiation therapy, conventional chemotherapy, and combination of radiation and bone marrow transplantation are the most well-known and established cancer therapies. Hormone therapy, immunotherapy and targeted therapy are more recently developed cancer treatments. Hormone therapy slows or stops the growth of breast and prostate cancers that use hormones to grow. Immunotherapy boosts the immune system to fight cancer. Targeted cancer therapy, involving small molecule drugs, monoclonal antibodies and immunotoxins targets specific proteins involved in tumor generation and progression and interferes with tumors grow and spread throughout the body (Baudino, 2015).

For example, recently approved immune checkpoint inhibitors: ipilimumab, nivolumab, and pembrolizumab have shown great anti-tumor effect in melanoma, lung cancer and renal

cancer. (Karlsson and Saleh, 2017;Kazaz and Oztop, 2017;Ross and Jones, 2017). Nivolumab and pembrolizumab target PD-L1 on tumor cells, while ipilimumab targets CTLA-4 on T cells, inhibiting PD-L1 and CTLA-4 interaction leading to activation of a T cell antitumor response (Karlsson and Saleh, 2017).

## **1.3 THE P53 TUMOR SUPPRESSOR**

### **1.3.1 Discovery of p53**

In 1979, p53 was first identified as a cellular protein physically complexed with SV40 virus large T-antigen, as shown by co-immunoprecipitation with anti-large T antibodies. The protein was named as p53 because the molecular mass was estimated to around 53 kDa on the basis of its migration in SDS-polyacrylamide gels, even though later the actual molecular mass of the human p53 protein was shown to be only 43.7 kDa; mouse p53 is even smaller (Lane and Crawford, 1979;Linzer and Levine, 1979;Levine and Oren, 2009). At the beginning, *TP53* was considered an oncogene due to p53 overexpression in various tumors and the ability of p53 cDNA to transform cells together with mutant *HRAS*. However, subsequent studies revealed that the p53 cDNA used in many experiments was in fact a mutant version of p53. Moreover, DNA sequencing of *TP53* in colorectal cancer and other tumors demonstrated frequent mutations. Thus, it became clear that wild type p53 is not an oncogene but rather a potent tumor suppressor gene (Levine and Oren, 2009).

### **1.3.2 Structure of p53**

The *TP53* gene, located on the short arm of chromosome 17 (17p13.1), contains 11 exons with a non-coding exon 1 and two cryptic exons (9 $\beta$  and 9 $\gamma$ ). The *TP53* gene encodes nine different mRNAs due to its alternative promoters at the N-terminus and alternative splicing at intron 2 and intron 9 (Joruiz and Bourdon, 2016). So far these nine different p53 mRNAs have been shown to encode 12 different p53 protein isoforms in humans (Bourdon et al., 2005;Marcel et al., 2010;Joruiz and Bourdon, 2016).

The wild type full-length p53 protein, with 393 amino acid residues (aa), is composed of two transactivation domains (TA1 and TA2), a proline-rich domain (PRD), a DNA binding domain (DBD), a nuclear localization signal (NLS), a oligomerisation domain (OD) and a regulatory domain (RD) (Joruiz and Bourdon, 2016).



**Figure 1. Schematic drawing of p53 with its various functional domains.** TA1, transactivation domains 1; TA2, transactivation domains 2; PRD, proline-rich domain; DBD, DNA binding domain; NLS, nuclear localization signal; OD, oligomerisation domain; NES, nuclear export signaling; RD, regulatory domain.

As shown in Figure 1, TA1 and TA2, located at N-terminus, are required for interaction with the transcriptional co-activators or co-repressors. This region is also critical for binding with MDM2. The proline rich domain (PRD) is important for the apoptotic activity of p53. The central core of p53 comprises the DNA binding domain (DBD), responsible for the specific DNA-binding function of p53, is critical for p53 –dependent transactivation function. The Nuclear localization signal (NLS) is essential for nuclear localization of p53. In the C-terminus of p53, the oligomerisation domain (OD) regulates the tetramerization process of p53, which is required for p53-dependent transactivation and p53-mediated growth suppression. The regulatory domain (RD), including a nuclear export-signaling sequence, is shown to play an important role in p53-mediated apoptosis, transcriptional regulation and DNA damage recognition (Choisy-Rossi et al., 1999; Batinac et al., 2003; Joerger and Fersht, 2010; Jorruiz and Bourdon, 2016).

### 1.3.3 p53 activation

Under normal conditions, p53 protein levels are low, mainly due to its inhibitor mouse double-minute 2 (MDM2) and mouse double-minute 4 (MDM4). MDM2, also known as human double-minute 2 (HDM2) in human, is an E3 ubiquitin ligase which targets N-terminus of p53, resulting in inhibition of transactivation domains as well as ubiquitin-dependent proteasomal degradation of p53. MDM4, also known as MDMX, has similar structure as MDM2, but without E3 ligase activity of its own. It binds to the N-terminus of p53 and inhibits p53 transactivation domains. However, MDM4 forms heterocomplexes with MDM2 and enhance p53 ubiquitination and degradation mediated by MDM2 (Brooks and Gu, 2006; Meek, 2015).

Various types of cellular stress, such as DNA damage, oncogene-induced hyper-proliferation, ("oncogenic stress"), oxidative stress, hypoxia, ultraviolet (UV) radiation, and viral infection, stabilizes p53 through releasing it from MDM2-mediated inhibition. ATM

(Ataxia telangiectasia mutated) and/or ATR (Ataxia telangiectasia and rad3 related) are activated in response to double and single-strand DNA breaks, respectively, leading to p53 phosphorylation and upregulation (Meek, 2015). Oncogene expression and abnormal cell proliferation stimulate the levels of alternative reading frame (ARF) protein, leading to p53 upregulation through direct binding to MDM2 (Meek, 2015). Besides, aberrant cell proliferation due to oncogene activation leads to a DNA damage response that involves p53 activation and forms a barrier against tumor development (Bartkova et al., 2005; Bartek et al., 2007).

### **1.3.4 p53 regulation**

p53 activity is regulated through various mechanisms including protein-protein interactions, post-translational modifications (PTMs), expression of p53 isoforms and cross-talking pathways. Apart from MDM2 and MDM4, which have been described previously, p53 can also be regulated by various proteins through protein-protein interactions. p53 can also be regulated by a wide range of PTMs, including ubiquitylation, phosphorylation, acetylation, methylation and sumoylation. So far, 12 different p53 isoforms have been shown to be expressed in different human cells and tissues. All these different p53 isoforms can undergo different PTMs and take part in protein-protein interactions. This creates a complex network that regulates p53 function (Meek, 2015).

### **1.3.5 p53 functions**

#### *1.3.5.1 Cell cycle arrest and DNA repair*

DNA damage activates cell cycle arrest, which allows DNA repair. DNA damage activates ATM/ATR pathway, resulting in activation of p53 and transactivation of its target genes and subsequent G1 and G2 arrest. *p21 (CDKN1A)*, the first p53 target gene to be discovered, encodes the cell cycle inhibitor p21. Induction of p21 expression causes both G1 and G2 arrest, as result of inhibition of cyclin E/CDK2 and cyclin B/CDK1 (Bunz et al., 1998; Li and Ho, 1998). Other p53 target genes, including 14-3-3 $\sigma$ , can also regulate cell cycle arrest (Hermeking et al., 1997). In addition, p53 is involved in base excision and nucleotide excision repair (Gotz and Montenarh, 1996; Soddu and Sacchi, 1997; Li and Ho, 1998).



#### 1.3.5.2 Apoptosis

p53 induces expression of a variety of genes involved in both extrinsic (death receptor) pathway and intrinsic pathway. Several of p53 target genes such as death receptor FAS, DR4 and DR5 are involved in extrinsic apoptotic pathway. FASL and TRAIL ligands bind to the FAS, DR4 and DR5 death receptors, triggering the recruitment of FAS-associated death domain (FADD), leading to activation of caspase-8, resulting in activation of effector caspases and apoptotic response (Fridman and Lowe, 2003; Amaral et al., 2010).

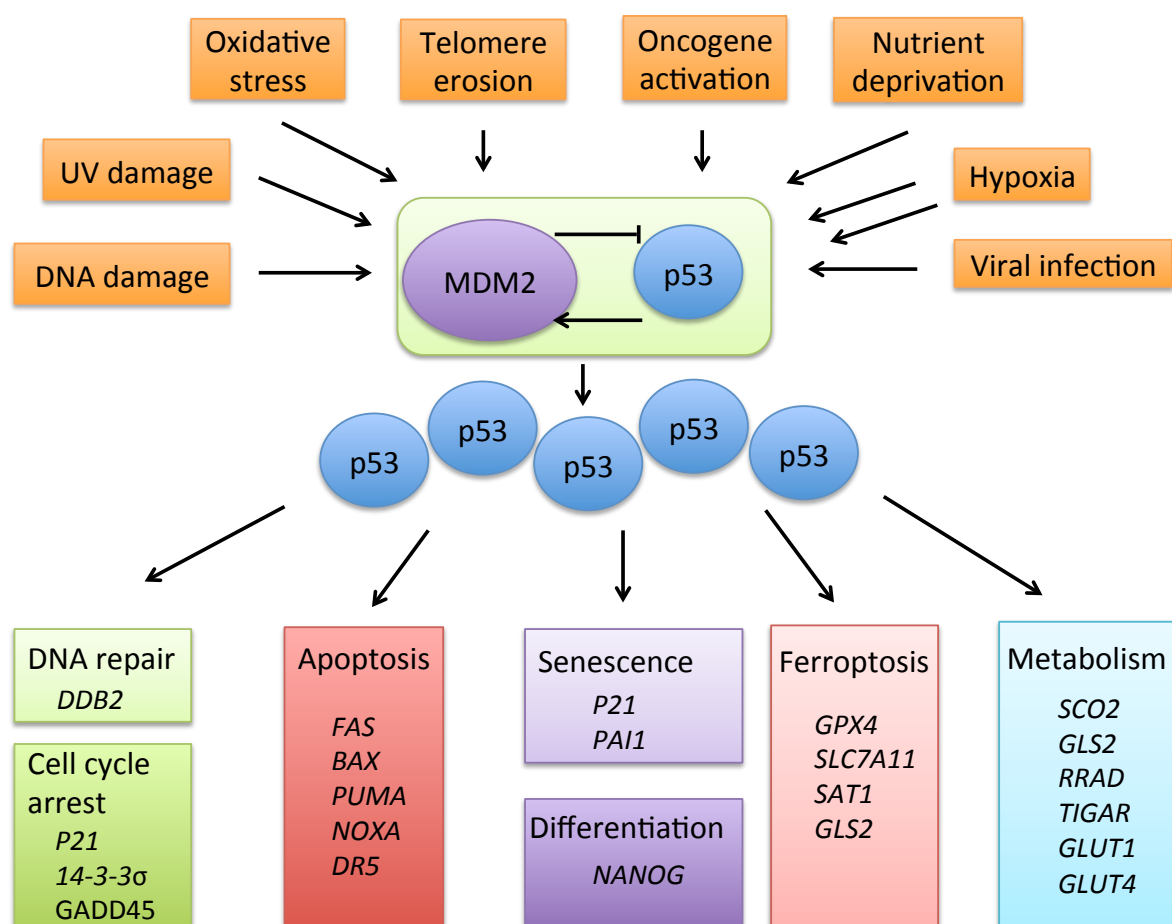
The intrinsic apoptotic (mitochondria) pathway, regulated by Bcl-2 family proteins, involves mitochondrial depolarization, translocation of cytochrome C from the mitochondrial intermembrane into the cytoplasm, apoptosome formation and activation of caspases. Bcl-2 family proteins can be divided into three groups: the anti-apoptotic proteins with structure similar to Bcl-2, e.g. Bcl-xL; the pro-apoptotic proteins structurally related to Bcl-2, e.g. Bax and Bak; the third group of the proteins are pro-apoptotic with only BH-3 domain called “BH3-only” proteins, e.g. Puma and Noxa. Several of p53 target genes are involved in this pathway, for example *BAX*, *PUMA* and *NOXA*. Puma and Noxa help Bax translocation to the surface of mitochondria. Bax oligomerizes and forms complex with Bax on the surface of mitochondria, as well as p53 forms complex with cyclophilin D in the mitochondrial inner membrane. These changes result in disruption of mitochondrial membranes and release of pro-apoptotic proteins such as cytochrome c and apoptosis-inducing factor (AIF) from mitochondria, which eventually activate caspase activity and apoptosis (Gotz and Montenarh, 1996; Li and Ho, 1998; Fridman and Lowe, 2003; Mihara et al., 2003; Amaral et al., 2010; Meek, 2015).

#### 1.3.5.3 Senescence

Cellular senescence, a form of irreversible cell cycle arrest, is induced by loss of replicative ability due to various stresses including dysfunctional telomeres, DNA damage, oxidative stress, oncogene activation and nutrients starvation. The senescent cells with a large and flattened morphology are still metabolically active, but do not proliferate. Several of p53 target genes including p21 play important role in activation of senescence. Senescence is induced by many chemotherapeutic agents that cause DNA damage and p53 activation. However, impairment of p53 function can inhibit drug-induced senescence and result in tumor progression. The ability of p53 to regulate senescence may contribute to p53-mediated tumor suppression *in vivo* (Qian and Chen, 2010; 2013).

### 1.3.5.4 Ferroptosis

p53 also regulates ferroptosis, an iron-mediated, caspase-independent programmed cell death requiring the accumulation of lipid peroxides and oxidative stress. This pathway was shown to be controlled by glutathione peroxidase 4 (GPX4), a glutathione-regulated lipid repair enzyme and to be regulated by p53 target genes *SLC7A11*, *SAT1* and *GLS2*. p53 transcriptionally repress *SLC7A11*, a component of the cystine/glutamate antiporter, system Xc-, resulting in reduced cystine import, leading to decreased glutathione production and increased reactive oxygen species (ROS). p53 can also transcriptionally activate glutaminase 2 (*GLS2*), and *SAT1*, resulting in increased ROS and lipid peroxides that are required for ferroptosis. Ferroptosis is a critical component of p53-mediated tumor suppression (Murphy, 2016; Gnanapradeepan et al., 2018).



**Figure 2. p53 activation and signaling pathways.**

#### 1.3.5.5 Metabolism

p53 is also involved in regulation of oxidative phosphorylation, glucose metabolism and lipid metabolism. Wild type p53 regulates cell metabolism by favoring mitochondrial respiration over glycolysis, mainly through promoting oxidative phosphorylation by transactivation of cytochrome c oxidase subunit 2 (*SCO2*), promoting glutaminolysis for mitochondrial respiration by transactivation of Glutaminase 2 (*GLS2*), and suppressing glycolysis by transactivation of glycolysis inhibitors RRAD and TIGAR, as well as transcriptionally repressing the glucose transporters *GLUT1* and *GLUT4*. In addition, p53 also suppressing glucose metabolism through directly binding and inhibition of the enzyme glucose-6-phosphate dehydrogenase (G6PDH) (Vousden, 2009;Gnanapradeepan et al., 2018).

WT p53 promotes fatty acid oxidation through transactivation carnitine palmitoyltransferase 1C (*CPT1C*) and Lipin 1 (*LPIN1*) while suppressing fatty acid synthesis through inhibition of G6PDH and sterol regulatory element-binding proteins (SREBP) via protein-protein interaction. Therefore, p53 and many of its target genes are involved in metabolic disease, such as obesity and diabetes (Vousden, 2009;Gnanapradeepan et al., 2018).

#### 1.3.5.6 Differentiation and Fertility

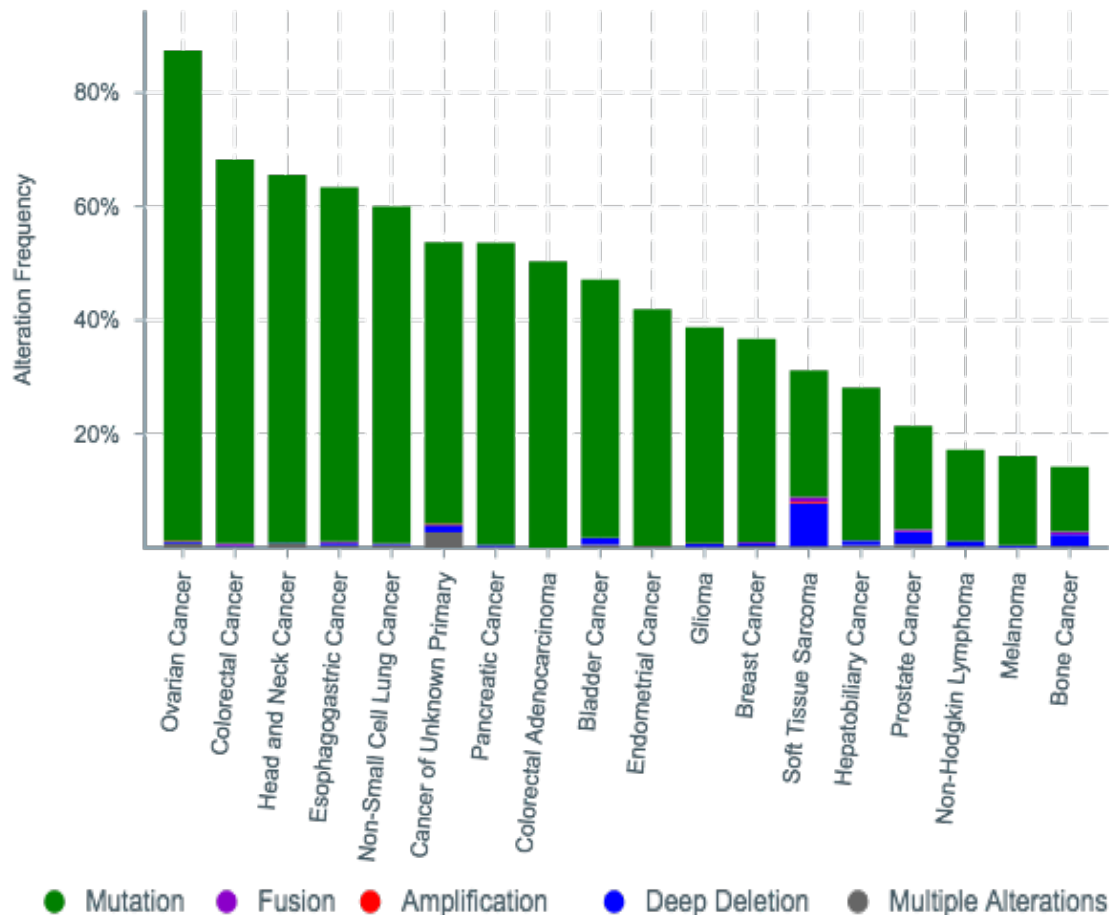
p53 suppresses stem cell self-renewal after DNA damage and induce stem cell differentiation, therefore eliminates tumorigenesis. Activation of p53 results in inhibition of reprogramming of both normal and cancer cells to induced pluripotent stem cells (iPSCs). p53 regulates embryonic implantation through transcriptional regulation of leukemia inhibitory factor (LIF). Loss of p53 genes in female mice leads to a significant decrease in fertility (Rotter et al., 1994;Li et al., 2013). Besides, p53 is also involved in inhibition of angiogenesis and metastasis (Song et al., 2015).

### 1.3.6 TP53 inactivation in cancer

*TP53* inactivation plays an key role in tumorigenesis and tumor progression. p53 inactivation by *TP53* mutation occurs in a large fraction of human tumors, as shown in Figure 2 (Cerami et al., 2012;Gao et al., 2013).

90% of *TP53* mutations are localized in the p53 core domain (residue 100 to 300). About 75% of *TP53* alterations are missense mutations, resulting in single amino acid substitutions in p53, leading to the synthesis of stable mutant proteins (Soussi and Wiman, 2015). It has

been shown that the gain-of-function of mutant p53 enhances cancer progression (Acin et al., 2011;Zhou et al., 2014). TP53 mutations are often but not always associated with poor therapy response and survival (Gudkov and Komarova, 2003;Robles and Harris, 2010;Said et al., 2013)



**Figure 3. TP53 alteration frequency in human cancer genomics.** Recorded in cBioPortal for cancer genomics. (<http://www.cbioportal.org/index.do>)

Approximately 10% TP53 alterations are nonsense mutations, changing an amino acid codon to a premature termination codon (PTC) (TAG, TGA, or TAA), resulting in premature termination of the protein translation process and expression of unstable truncated proteins (Linde and Kerem, 2008). In addition, mRNAs with PTCs are usually expressed at low levels due to degradation by nonsense-mediated decay (NMD) (Isken and Maquat, 2008;Lykke-Andersen and Jensen, 2015). R213X is the most common TP53 nonsense mutation, representing around 19% of all TP53 nonsense mutations and approximately 2 % of all TP53 mutations in human tumors.

In addition to *TP53* mutation, wild type p53 can be inactivated in tumors by the cellular antagonists MDM2 and MDM4, and by viral proteins such as HPV E6, SV40 large T and adenovirus E1B55K (Levine, 2009; Tsai et al., 2009).

## 1.4 P53-TARGETED CANCER THERAPY

### 1.4.1 Wild type p53-targeted cancer therapy

p53 inactivation is a universal feature of human cancer cells. Around 50% of cancers carry wild type p53 which can be inactivated through various mechanisms. The E3 ubiquitin ligase MDM2 is the one of the most important regulators of p53. Negative regulation of wild type p53 through overexpression of MDM2/MDM4 frequently occurs in human cancers with wild type p53. Many compounds were identified to target the interaction between wild type p53 and MDM2/MDM4. For example, nutlins and RITA have been shown to inhibit the interaction between MDM2 and p53, and induce p53 target genes as well as p53-mediated cell cycle arrest and apoptosis (Issaeva et al., 2004; Doggrell, 2005; Trino et al., 2016). SJ-172550 inhibits the interaction between MDM4 and p53, leading to p53-dependent apoptosis (Bista et al., 2012). Besides, antisense oligos and siRNAs have been used to inhibit MDM2 and HPV E6 to induce rapid and effective p53 response (Jiang and Milner, 2002; Zhang et al., 2005). Several p53-Mdm2 inhibitors are currently or have been tested in clinical trials, including RG7112, GO5503781, MI-773 and DS-3032b (Khoo et al., 2014).

### 1.4.2 Missense mutant p53-targeted cancer therapy

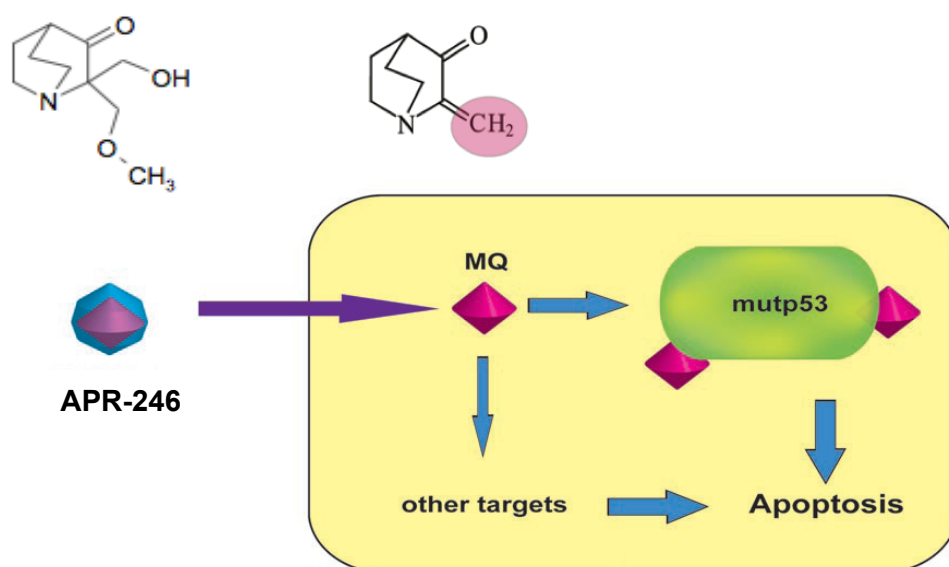


Figure 4. APR-246 is converted to MQ inside cells. Adapted from Bykov *et al.*, Cell Cycle 2009.

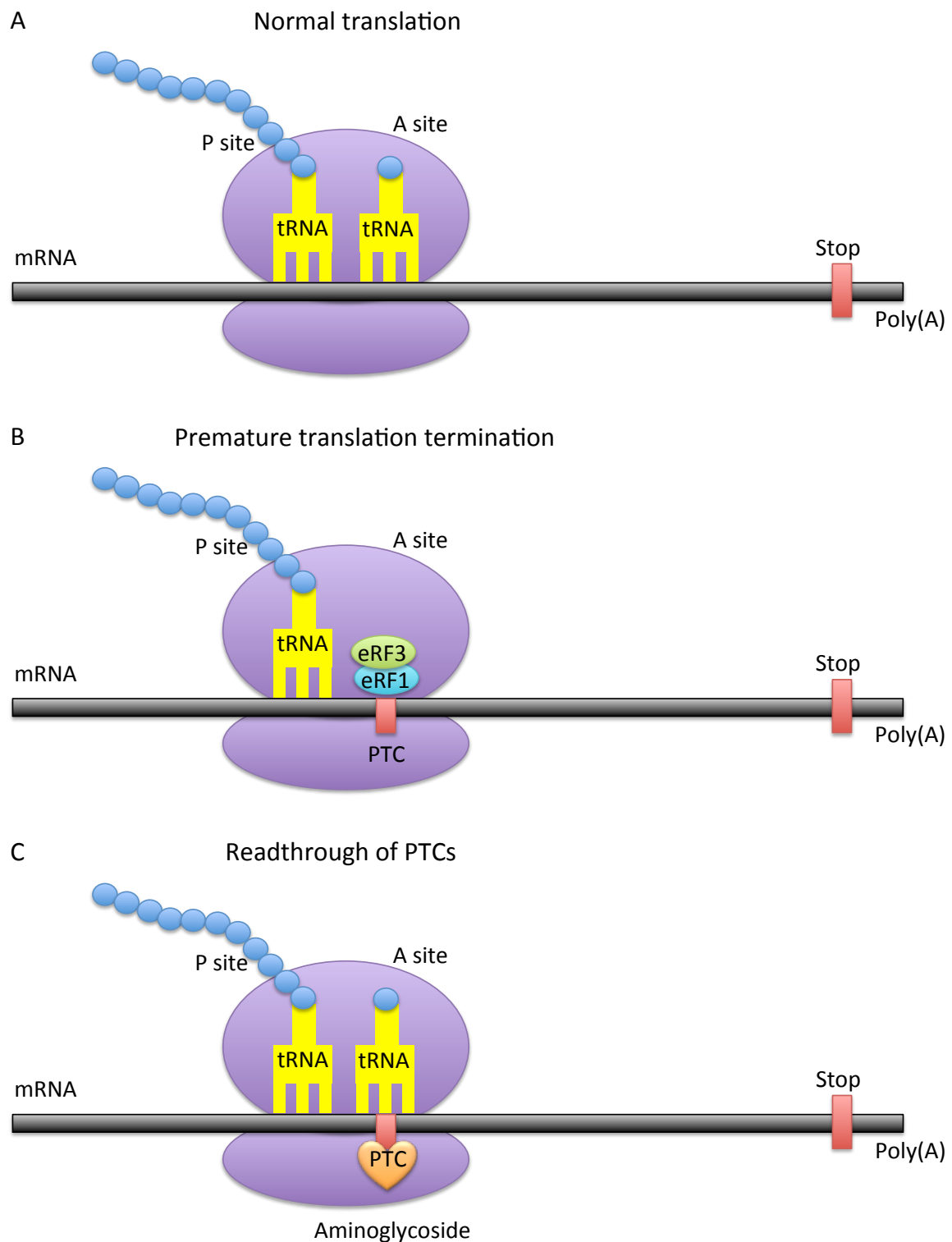
Around 50% of human tumors carry *TP53* mutations. As described above, the majority of *TP53* (75%) alterations are missense mutations. A lot of efforts have been made to identify small molecules that can reactivate mutant p53. APR-246 is the most well-studied compound in this category. The small molecule PRIMA-1 was identified in cell-based screen of the National Cancer Institute (NCI) Diversity set of compounds for substances inducing mutant p53-dependent growth suppression in tumor cells. PRIMA-1 induced p53 target genes and p53-mediated apoptosis in cancer cells (Bykov et al., 2002a; Bykov et al., 2002b). APR-246 (PRIMA-1Met) is a more potent analog of PRIMA-1. Both APR-246 and PRIMA-1 are converted to methylene quinuclidinone (MQ). MQ is a potent electrophile and Michael acceptor and responsible for the biological effects of APR-246. APR-246 inhibits the growth of cancer cells in culture as well as xenograft tumors in mice (Bykov and Wiman, 2014). Furthermore, APR-246 synergizes with DNA-damaging agents such as adriamycin and cisplatin to induce apoptosis in cancer cells (Bykov et al., 2005; Fransson et al., 2016). Although the exact mechanism is not fully understood, MQ binding to cysteines in p53 promotes refolding of p53 to a "wild type-like" conformation. Cysteine 277 is a prime binding site for MQ and required for MQ-mediated thermostabilization of the p53 core domain (Zhang et al., 2018).

APR-246 has been investigated in a phase I/IIa clinical trial in 22 patients with prostate cancer and different types of hematologic malignancies (clinicaltrials.gov Identifier: NCT00900614) (Medicine, 2011). The treatment was well tolerated. The most common adverse effects were fatigue, dizziness, headache and confusion. This study and an extension study with another 10 patients also showed clinical effects in five out of six patients carrying mutant *TP53* (Lehmann et al., 2012).

Currently, APR-246 is undergoing a phase II proof-of-concept randomized clinical trial in patients with recurrent high-grade serous ovarian cancer, a tumor type with a *TP53* mutation frequency of around 95% (Clinical-Trials.gov Identifier: NCT02098343). The study investigates APR-246 in combination treatment with carboplatin and pegylated doxorubicin. Meanwhile, a phase Ib/II clinical trial of APR-246 in combination with the cytosine analogue azacitidine in patients with myelodysplastic syndrome (MDS) carrying *TP53* mutations has been initiated. In addition, a phase Ib/II clinical trial of APR-246 in combination with cisplatin and 5-fluorouracil in patients with esophageal cancer has started.

Several other small molecules have been shown to restore wild type p53 conformation and function to missense mutant p53, including MIRA, PK11007, ZMC1 and stictic acid (reviewed by Bykov et al., 2018)

### 1.4.3 Nonsense mutant p53-targeted cancer therapy



**Figure 5. The effect of readthrough drug aminoglycoside on protein translation.**

Approximately 10% *TP53* mutations are nonsense mutations, which give rise to premature termination codons (PTCs). *TP53R213X* is the most common nonsense mutation. During the normal translation process, ribosomes move along the mRNAs while the codons on the

mRNAs pair-match with anticodons on tRNAs, linking amino acids into proteins until arriving at the stop codons. However, when a ribosome encounters a PTC due to nonsense mutation, the translation process is terminated prematurely, due to no tRNA pair-match with PTC. At the same time eukaryotic release factors (eRF1 and eRF3) are recruited and unstable truncated protein is released (Linde and Kerem, 2008). mRNAs containing premature termination codons (PTCs) activate nonsense-mediated mRNA decay (NMD) (Isken and Maquat, 2008; Lykke-Andersen and Jensen, 2015). Nonsense mutations are not only causative in genetic diseases such as cystic fibrosis and Duchenne muscular dystrophy, but also common in various types of tumors (Mort et al., 2008; Prokofyeva et al., 2013; Wei et al., 2014).

Induction of translational readthrough is an interesting potential therapeutic strategy for genetic diseases and cancers caused by nonsense mutations. Aminoglycoside antibiotics G418 and gentamicin have been shown to suppress PTCs in a variety of genes, including *TP53*, leading to upregulation of p53 target genes and p53-dependent cell death (Floquet et al., 2011; Bidou et al., 2017). Gentamycin B1, a minor gentamicin component, was shown to be more potent readthrough inducer (Baradaran-Heravi et al., 2017). However, treatment with aminoglycosides can lead to severe nephrotoxicity and ototoxicity, which makes them less attractive for long-term treatment at high doses.

There are also a few non-aminoglycosides readthrough-inducing compounds. The oxadiazole Ataluren (PTC124) was identified in a high-throughput screen using a firefly luciferase-based readthrough reporter. However, its molecular mechanism remains controversial (Sheridan, 2013) and data suggest that Ataluren stabilizes the luciferase protein (Auld et al., 2010). Clinical studies with Ataluren in cystic fibrosis and Duchenne muscular dystrophy have not shown any striking beneficial effect (Zainal Abidin et al., 2017). Nonetheless, Ataluren recently obtained conditional approval by the European Medicines Agency (EMA) for treatment of Duchenne muscular dystrophy (Mullard, 2014).

NMD inhibitors NMDI14 and amlexanox were also shown to promote readthrough of PTCs. In addition, combination of readthrough reagent and NMD inhibitors showed synergistic effects on restoration of full-length p53 protein as well as induction of p53 target gene (Gonzalez-Hilarion et al., 2012; Martin et al., 2014).



## 1.5 REDOX HOMEOSTASIS AND CANCER THERAPY

Redox homeostasis is the balance between reactive oxygen species (ROS) generation and ROS elimination by antioxidant systems in cells. Regulation of redox homeostasis plays a vital role in maintaining normal cellular functions and ensuring cell survival (Gorrini et al., 2013; Tong et al., 2015).

Cancer cells are characterized by elevated aerobic glycolysis (the Warburg effect) and high levels of oxidative stress due to increased ROS generation in order to hyper-activate cell signaling pathways that promote cancer cell proliferation, survival and adaptation to the tumor microenvironment, e.g. hypoxia. These high levels of ROS are counteracted by elevated antioxidant systems in cancer cells (Gorrini et al., 2013; Glasauer and Chandel, 2014; Tong et al., 2015).

The thioredoxin (Trx) system and the glutathione (GSH) system are the two major systems that maintain cytosolic redox homeostasis. The Trx system comprises of Trx, thioredoxin reductase (TrxR) and the cofactor NADPH, and the GSH system consists of GSH, glutathione reductase (GR), glutaredoxin (Grx), and NADPH. Nuclear factor erythroid 2-related factor 2 (NRF2) is the master regulator of antioxidant responses which regulates the expression of multiple enzymes involved in several antioxidants pathways including both Trx and GSH systems as well as other antioxidant enzymes, e.g. SOD (Cebula et al., 2015; Xu et al., 2016).

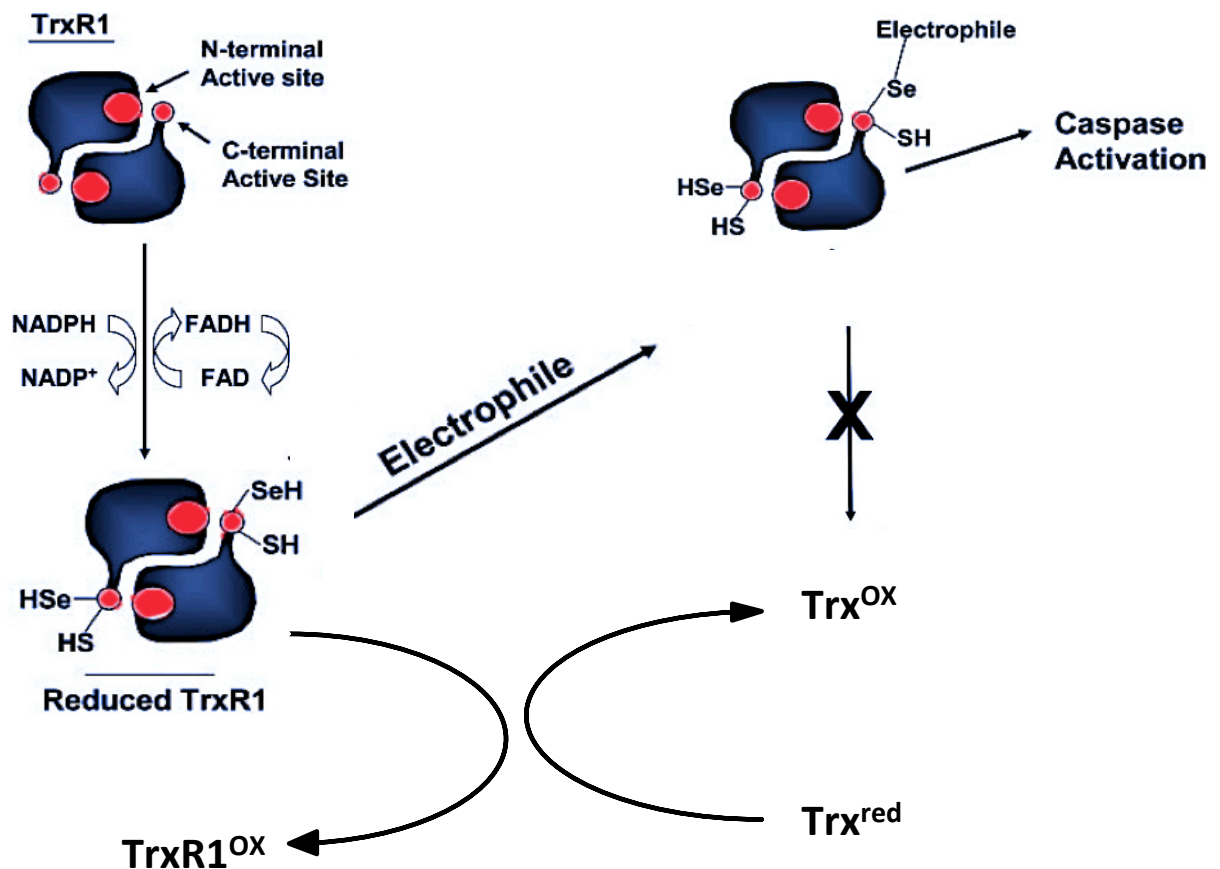
TrxR is the only known enzyme to reduce Trx, with three isoforms in human including TrxR1, TrxR2 and TrxR3. TrxR1 is the major form of TrxR expressed in most tissues with the location mainly in cytosol, and TrxR2 is mainly located in mitochondria. TrxR3 is only found in various tissues at very low levels (Mustacich and Powis, 2000; Conrad et al., 2004; Cebula et al., 2015).

Mammalian TrxR1 exists as homodimer with a Sec in its C-terminal active domain in each monomer. During the catalytic process, the N-terminal active site of TrxR1 oxidases NADPH and transfers electrons to the C-terminal active site of the other monomer. The reduced C-terminal active site with a selenothiol motif subsequently reduces various substrates including oxidized Trx, as well as synthesized chemical dithionitrobenzoic acid

(DTNB). Reduction of DTNB results in production of thionitrobenzoic acid (TNB), whose color can be measured at 412nm with a plate reader or spectrophotometer. However, some substrates such as certain quinones, e.g. organic compound 5-hydroxy-1, 4-naphthoquinone

(juglone) can be reduced directly by the N-terminal active site of TrxR1 (Conrad et al., 2004; Peng et al., 2012; Peng et al., 2013; Xu et al., 2016).

TrxR1 through reduced Trx1 plays a very important role in DNA synthesis and protection against redox stress. Besides, TrxR1 has also been shown to regulate p53 redox status. Inhibition of TrxR1 in yeasts and in human cells was shown to induce disulfides in p53 and inactivate DNA binding but not affect p53 protein levels. In addition, TrxR1 is very important for certain signaling events required for embryonic development. Knockdown of TrxR1 through gene deletion results in embryonic death. However, knockdown of TrxR1 in cells and tissues does not necessary exhibit apparent phenotype. The DNA synthesis and antioxidant defense in TrxR1 deficient cells is compensated by elevated NRF2 and GSH systems (Mustacich and Powis, 2000; Hedstrom et al., 2009; Bykov et al., 2016).



**Figure 6. Gain of pro-oxidant function of TrxR1 after modification by an electrophile at Sec C-terminal active motif.** Adapted from Cassidy *et al.*, Carcinogenesis 2006.

Mammalian TrxR1, in its C-terminal active domain, contains selenocysteine with low pKa and higher nucleophilicity that is an easily accessible target for electrophiles. TrxR1, with Sec inhibited in C-terminal active domain by electrophiles such as cisplatin while NADPH oxidase activity preserved at N-terminal active domain, results in not only inhibition of reducing activity but also gain of pro-oxidant function that induces massive ROS production and caspase activation which ultimately leads to extensive cancer cell death. Cancer cells usually express higher level of TrxR1, and targeting TrxR1 shows higher toxicity in cancer cells rather than in normal cells, which make it a promising anticancer strategy (Peng et al., 2012; Peng et al., 2013; Cebula et al., 2015).



## **2 AIMS OF THE THESIS**

The general aims of the thesis were to identify novel compounds that can induce translational readthrough of nonsense mutant *TP53* and further investigate the molecular mechanisms of the missense mutant p53-targeting compound APR-246. These studies may open avenues for novel p53-targeted cancer therapy.

### **Specific aims**

#### **Paper I**

To investigate whether APR-246 targets the selenoprotein thioredoxin reductase 1 (TrxR1) and whether this contributes to APR-246-induced tumor cell death.

#### **Paper II**

To explore combination treatment with aminoglycosides and Mdm2 inhibitors to induce translational readthrough of nonsense mutant *TP53*.

#### **Paper III**

To identify novel nonsense mutant *TP53*-targeting compounds through data mining.

#### **Paper IV**

To perform high-throughput screening to identify novel compounds that induce translational readthrough of nonsense mutant *TP53*.



## 3 RESULTS AND DISCUSSION

### 3.1 PAPER I

#### **APR-246/PRIMA-1<sup>MET</sup> inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase**

We previously showed that both PRIMA-1 and APR-246 are converted into the active compound MQ that covalently binds to Cys residues in mutant p53 and restore wild type p53 conformation and function to mutant p53, leading to induction of p53 target genes and p53-mediated cancer cell death. We would like to investigate whether MQ also has other targets in tumor cells. One potential target highly related to cancer is thioredoxin reductase 1 (TrxR1), an important regulator of redox balance in cells, which catalyzes the reduction of thioredoxin using NADPH. Since mammalian TrxR1 contains a selenocysteine (Sec) in its active motif and Sec is significantly more reactive than Cys due to its higher nucleophilicity.

To investigate whether APR-246 targets TrxR1 and affects its function, we first tested if APR-246 affects the function of TrxR1 *in vitro*. It revealed that both pre-heated APR-246 and MQ strongly inhibited the reducing activity of TrxR1 while maintained its ability to consume NADPH. In addition, the Sec to Cys variant of TrxR1 was much more resistant to inhibition induced by APR-246 and MQ, indicating that the Sec residue of the TrxR1 was the primary target of MQ during the inhibition of the TrxR1.

To analyze if APR-246 also inhibits the activity of TrxR1 in cells, various cancer cells with different p53 status were treated with APR-246 at different concentrations for different times periods. This showed that TrxR1 activity strongly decreased by APR-246 treatment independent of p53 status.

In order to test whether the inhibition of TrxR1 contributes to cell death induced by APR-246, we used siRNAs to downregulate the expression of TrxR1. Data revealed that APR-246 induced substantial cell death in cancer cells with p53 missense mutation but much milder cell death in p53 null cancer cells. Knockdown of TrxR1 did not induce any substantial cell death by itself. However, it partially rescued cell death induced by APR-246 in both cell lines, independent of p53 status. These results suggested that mutant p53 was the main target for APR-246 induced cancer cell death, and TrxR1 was the one of the secondary targets that contributes to APR-246 mediated cancer cell death.

How the inhibition of TrxR1 by APR-246 affected reactive oxygen species (ROS) generation was also examined. The results uncovered that APR-246 strongly induced production of ROS in cancer cells with p53 missense mutation while it only mildly upregulated ROS production in p53 null cancer cells. Knockdown of TrxR1 by siRNA did not induce substantial ROS generation by itself, but strongly attenuated ROS production induced by APR-246. The results indicated that TrxR1 contributed significantly to ROS induced by APR-246.

To sum up, our study uncovered that TrxR1 is a target of APR-246, which contributes to APR-246-induced ROS induction and cancer cell death. Inhibition of TrxR1 by APR-246 in addition to its ability to restore wild type function to missense-mutated p53 produced an additive effect on disrupting the cellular redox balance and accelerated apoptosis signaling cascade. Targeting both TrxR1 and missense mutant p53 may also reduce the risk of resistance to treatment.



### 3.2 PAPER II

#### **Synergistic rescue of nonsense mutant tumor suppressor p53 by combination treatment with aminoglycosides and Mdm2 inhibitors.**

The tumor suppressor p53 is frequently inactivated by *TP53* mutations. Approximately 10% of *TP53* mutations are nonsense mutations resulting in the expression of unstable truncated p53 proteins. Aminoglycosides G418 and gentamicin have been shown by previous study to induce readthrough of PTCs and expression of full-length proteins including full-length p53. However, the nephrotoxicity and ototoxicity of aminoglycosides limit its clinical usage.

In this study, the ability of aminoglycosides to induce readthrough of PTCs in TP53 was first examined in HDQ-P1 cell carrying endogenous R213X nonsense mutant p53 and H1299 transfected with plasmid containing p53 coding sequence with a nonsense mutation at codon 213 or with plasmid containing the first 213 codons of R213X p53 fused in frame with the EGFP coding sequence. This showed that G418 induced readthrough of PTC (R213X) in all tested cell lines leading to expression of full-length protein p53 as well as R213X $\Delta$ C-EGFP fusion protein. In addition, G418 induced p53 mRNA in a time dependent manner. Gentamicin also induced p53 mRNA and expression of full-length p53 protein, although much less efficiently.

To further study whether the full-length p53 protein induced by aminoglycosides in cancer cells is transcriptional active, p53 target genes were analyzed at both protein and mRNA levels. The results revealed that G418 and gentamicin induce p53 target genes at both mRNA and protein levels, indicating the induced full-length p53 protein is transcriptional active.

With the question whether the restored full-length p53 could be further enhanced by combination treatments. We analyzed the aminoglycosides in combination with proteasome inhibitor bortezomib and p53-Mdm2 inhibitors, including nutlin-3a and MI-773. This showed that the combination treatments enhanced the expression of full-length p53 protein. The combination treatment with nutlin-3a and aminoglycosides showed strongly synergistic effect on induction of both mRNA and full-length protein of p53 as well as p53 target genes.

Last but not the least, we explored if the combination treatment potentiates p53 mediated cellular response. The results showed that G418 induced nonsense mutant p53 dependent

growth inhibition, cell death and cell cycle arrest. G418 in combination with nutlin-3a enhanced growth inhibition and showed synergistically enhanced cancer cell death.

In conclusion, our study demonstrates that combination treatment with aminoglycosides and p53-Mdm2 inhibitors provide strong synergistic effects on expression of full-length p53 protein, induction of p53 target genes as well as p53-mediated cancer cell suppression. This study may contribute to future cancer therapy that targets nonsense mutant TP53 and restores wild type p53 anti-cancer activity.

### 3.3 PAPER III

#### **Rescue of nonsense mutant p53 in human tumor cells by compounds identified through data mining**

National Cancer Institute (NCI) provides data on the growth-inhibition (GI) effect of many chemical compounds in 60 different human cancer cell lines. We performed an analysis based on NCI-60 database, in order to identify compounds that specifically induce growth inhibition in cancer cells with *TP53* nonsense mutations rather than cancer cells carrying other *TP53* mutations or wild type *TP53*. We identified 28 potential interesting compounds, including the known anticancer drug 5-fluorouracil (5-FU), based on analysis of 47,000 compounds.

We examined 18 compounds available from NCI out of the identified 28 compounds. The results revealed that 5 compounds, designated as MZ21, MZ23, MZ26, MZ30 and MZ32, were able to induce full-length p53 protein in cancer cells with p53 nonsense mutation R213X.

Moreover, 5-FU treatment restored substantial expression of full-length p53 in both endogenous p53 nonsense-mutant HDQ-P1 cancer cells and cancer cells H1299 R213X, H1299 cells stably transfected with plasmid carrying p53-coding sequence with a PTC (R213X). 5-FU also induced mRNAs of p53 and p53 target genes in HDQ-P1 and H1299-R213X cells but not H1299 empty vector cells indicating the p53 target gene induction by 5-FU is p53-dependent and suggesting that the restored full-length p53 protein is transcriptionally active.

In addition, we also tested 5-FU in H1299 cells transfected with a plasmid carrying only the first 213 codons of R213X p53 fused in frame with EGFP (H1299-R213X $\Delta$ C-EGFP). This showed that 5-FU restored expression of p53 R213X $\Delta$ C-EGFP fusion protein.

In summary, 5-FU restored expression of full-length p53 protein and p53 transcriptional activity in cancer cells with p53 nonsense mutation. However, the underlying mechanisms remains unknown. We provide several possible reasons including direct stabilization of p53 mRNA, stabilization of p53 protein and translational readthrough of PTC. Further study is required to elucidate the exact molecular mechanisms. The uncovered ability of 5-FU to restore expression of full-length p53 and p53 function in cancer cells carrying *TP53* nonsense mutation may open new therapeutic applications for 5-FU. Several other compounds identified from our NCI database mining also showed promising ability to

induce expression of full-length p53 in cancer cells with *TP53* nonsense mutation. The identification of small molecules that can rescue full-length p53 expression in cancer cells with nonsense mutant TP53 may ultimately allow the design of novel anticancer drugs.

### 3.4 PAPER IV

#### **High-throughput screening for identification of novel compounds that induce translational readthrough of nonsense mutant p53**

In this study, we first established a high-throughput screening assay based on p53-immunofluorescence staining in HDQ-P1 breast cancer cells carrying a homozygous nonsense mutation in *TP53* at codon 213. We screened 33,000 compounds from different libraries and selected 47 compounds designated as potential candidate readthrough-inducing compounds (CRICs). We also screened a library of anti-infectious compounds (130 molecules) in HDQ-P1 cells by FACS. We selected 18 compounds that induced more p53-positive cells than G418 as CRICs for further study. In total, we selected 65 compounds as CRICs based on these screens.

The CRICs were further tested by ELISA in H1299-R213X $\Delta$ C-FLAG cells and by Western blotting in H1299-R213X to confirm their ability of restoring expression of full-length protein. Several CRICs including CRIC 3, 44, 45, 47, 50, 53 and 58 induced both fusion protein p53 R213X $\Delta$ C-FLAG in H1299-R213X $\Delta$ C-FLAG cells and full-length p53 protein in H1299-R213X cells.

The observed ability of CRIC 44, 45, 47, 50, 53 and 58 to induce full-length p53 protein expression in H1299-R213X cells is consistent with our previous p53 immunostaining data in HDQ-P1 cells. CRIC 44, 45, 47, 50, 53 and 58 induced p53 immunostaining in a dose-dependent manner in HDQ-P1 cells. Further studies are required to uncover the mechanisms underlying the ability to restore expression of full-length p53 in cancer cells with *TP53* nonsense mutation and to elucidate the biological activity of the restored full-length p53 protein. We believe that our study will benefit nonsense-mutant p53-targeted cancer therapy.



## 4 CONCLUSIONS

**Paper I.** In this study, we show that APR-246 converted to MQ inhibits the reducing activity of both recombinant TrxR1 in vitro and cellular TrxR1 in cells independent of p53 status. A Sec-to-Cys variant of TrxR1 is resistant to inhibition induced by APR-246 indicating that Sec residue is the target of MQ. Inhibited TrxR1 by APR-246 maintains its ability to oxidize NADPH resulting in massive ROS induction. Knockdown of TrxR1 attenuates APR-246-induced ROS induction and cell death independently of p53 status.

**Paper II.** In this paper, we reveal that aminoglycosides G418 and gentamicin induce strong translational readthrough of PTC (R213X) in *TP53*, which results in expression of functional full-length p53 protein leading to induction of p53 target genes as well as p53-mediated growth inhibition and cell death in cancer cells. More important, the combination treatment with aminoglycosides and p53-Mdm2 inhibitors synergistically increases expression of full-length p53 protein and largely potentiates p53-mediated biological response.

**Paper III.** In this study, from data mining of the NCI database, we first identified 28 compounds as potential agents targeting nonsense-mutant p53 cancer cells. Among these, the known anticancer drug 5-FU and 5 other compounds without any known anti-cancer activity were shown to induce full-length p53 protein in nonsense-mutant p53 cancer cells. Full-length p53 protein induced by 5-FU is transcriptionally active as assessed by upregulation of several p53 target genes.

**Paper IV.** In this study, from a high-throughput screening based on p53 immunostaining and a FACS based screening, we selected 65 compounds as potential candidate readthrough-inducing compounds (CRICs). Among these, 7 compounds were shown to induce full-length p53 protein in nonsense-mutant p53 cancer cells.

## 5 FUTURE PERSPECTIVES

With the aim to identify future nonsense-mutant p53 targeted therapeutic agents with low toxicity profile, we did data mining of NCI database and high-throughput screening as well as FACS based screening. So far, several compounds including 5-FU were shown to induce full-length p53 in cancer cells with *TP53* nonsense mutation.

5-FU restored full-length p53 protein is transcriptionally active as assessed by upregulation of p53 target genes in a p53 dependent manner. We would like to further investigate whether 5-FU causes p53-mediated cellular response including cell death, cell cycle arrest and apoptosis. Except 5-FU, we are not sure if induced full-length p53 protein in cancer cells with *TP53* nonsense mutation by other compounds is functional. We will further investigate this issue.

The mechanisms underlay induced full-length p53 in cancer cells with *TP53* nonsense mutation by these compounds are still poorly understood. We provide several possible mechanisms. The first possible reason of upregulated full-length p53 expression may be a direct stabilization of p53 mRNA through inhibition of nonsense-mediated mRNA decay (NMD), a process that normally degrades mRNAs with a PTC. Inhibition of NMD would lead to enhanced p53 mRNA levels and possibly higher levels of full-length p53 as a result of spontaneous readthrough. The known NMD inhibitor amlexanox has been shown to rescue nonsense mutations in *TP53*, *DMD* and *CFT* (Gonzalez-Hilarion et al., 2012).

Another plausible reason for the observed upregulation of p53 levels could be stabilization of full-length p53 due to low levels of spontaneous readthrough. To address this possibility, we will test if these compounds can induce p53 expression in cells carrying wild type *TP53* such as HCT116 and MCF7.

A third possible mechanism for induction of full-length p53 protein by these compounds is induction of translational readthrough. It is very important to test if these compounds can indeed induce translational readthrough of PTC. To examine this, we will test them in a p53-independent context using a relevant reporter construct, such as EGFP or RFP containing an internal PTC. Moreover, we will also investigate if these compounds can rescue expression of full-length protein from other nonsense mutant genes, e.g. nonsense mutant *APC* or *RBI*.

Further studies are required to elucidate which one of these mechanisms, or a combination of several, is responsible for induction of full-length p53 by these compounds respectively.



If we can confirm one or several compounds with the ability to induce translational readthrough of PTCs, We will apply structure-activity relationship (SAR) analysis to identify critical chemical structures with the aim of improving readthrough efficacy and other features of relevance for drug development.

Besides, combination treatment with novel readthrough inducing compounds and p53-Mdm2 inhibitors may also improve readthrough efficacy and thus allow reducing the toxicity of the treatment. Our long-term goal is to take the most promising readthrough compound and/or combination treatments through preclinical development and ultimately clinical trials.

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